recruitment of Foxp3$^+$ regulatory T cells into tumors [63]. Moreover, MFG-E8 contributes to tumor growth, resistance to cell death signals, and epithelial–mesenchymal transition by activating phosphatidylinositol-3-kinase (PI3K)–Akt1 signals and inducing twist1 [64]. MFG-E8 also promotes cancer stem cell activation in cooperation with IL-6 by stimulating Stat3 and sonic Hedgehog pathways [36] (Figure 3). Consistent with its broad role in tumorigenic signals, pharmacological blockade of MFG-E8 improved the antitumor effects of various anticancer agents, including cisplatin, doxorubicin, dacarbazine, the epidermal growth factor (EGF) kinase inhibitor, and the anti-VEGF2 receptor antagonist [65]. Thus, MFG-E8 serves as a major mediator that links immune tolerance with tumor chemoresistance via recognition of apoptotic tumor cells. Emerging evidence has demonstrated that other phagocytic molecules also exert negative regulation of tumor chemosensitivity. Mer-tyrosine kinase (MerTK) serves as a phagocytic receptor for apoptotic cells via recognition of Gas-6, and may promote tumorigenic activities by regulating tumor angiogenesis and migratory activity [66,67]. In addition, the macrophage scavenger receptor SR-A is induced at high levels on tumor-associated macrophages, which contribute to invasive and metastatic activities of ovarian and pancreatic cells [68]. Thus, the innate recognition of apoptotic cells by phagocytic receptors has an essential function in generating chemoresistant conditions in tumor microenvironments.

Certain subsets of chemotherapeutic agents such as oxaliplatin, anthracycline, and methotrexate, etc., have an intrinsic ability to trigger ICD of tumor cells [46,69]. ICD causes cell surface translocation of calreticulin (CRT) on tumor cells. CRT serves as an 'eat me signal' by interacting with CD91 on myeloid cells [70]. ER stress responses caused by the hyperploid phenotype of tumor cells promote the translocation of CRT to the cell surface, which is correlated with tumor immunogenicity [71]. The CRT-mediated recognition of stressed tumor cells facilitates immunogenic phagocytosis by myeloid cells, leading to efficient antigen presentation and cross-priming of cytotoxic T lymphocytes [72].

Interestingly, CD47 expressed on viable or stressed tumor cells serves as a negative regulator of the CRT-mediated 'eat me' signal by interacting with signal regulatory protein α (SIRPα) [73]. Moreover, CD47-mediated inhibition of phagocytic pathways is responsible for the ability of acute myeloid leukemia stem cells to evade immunosurveillance. Pharmacological targeting of the CD47–SIRPα interaction facilitates the digestion of hematological and solid tumor stem cells by phagocytes and aids efficient tumor eradication [74].

Together, chemotherapeutic agents trigger two modes of cellular stress in tumor cells, each of which leads to distinct immunogenic consequences: a few cytotoxic agents generate ICD and mediate 'immunogenic' phagocytosis. This facilitates efficient processing and presentation of tumor rejection antigens to cytotoxic T lymphocytes through CRT–CD19-mediated recognition systems. By contrast, most chemotherapeutic agents have little ability to generate ER stress-related signals and induce ICD. These agents instead lead to 'tolerogenic' phagocytosis through the MFG-E8, Gas-6, or SR-A-dependent recognition of PS on apoptotic tumor

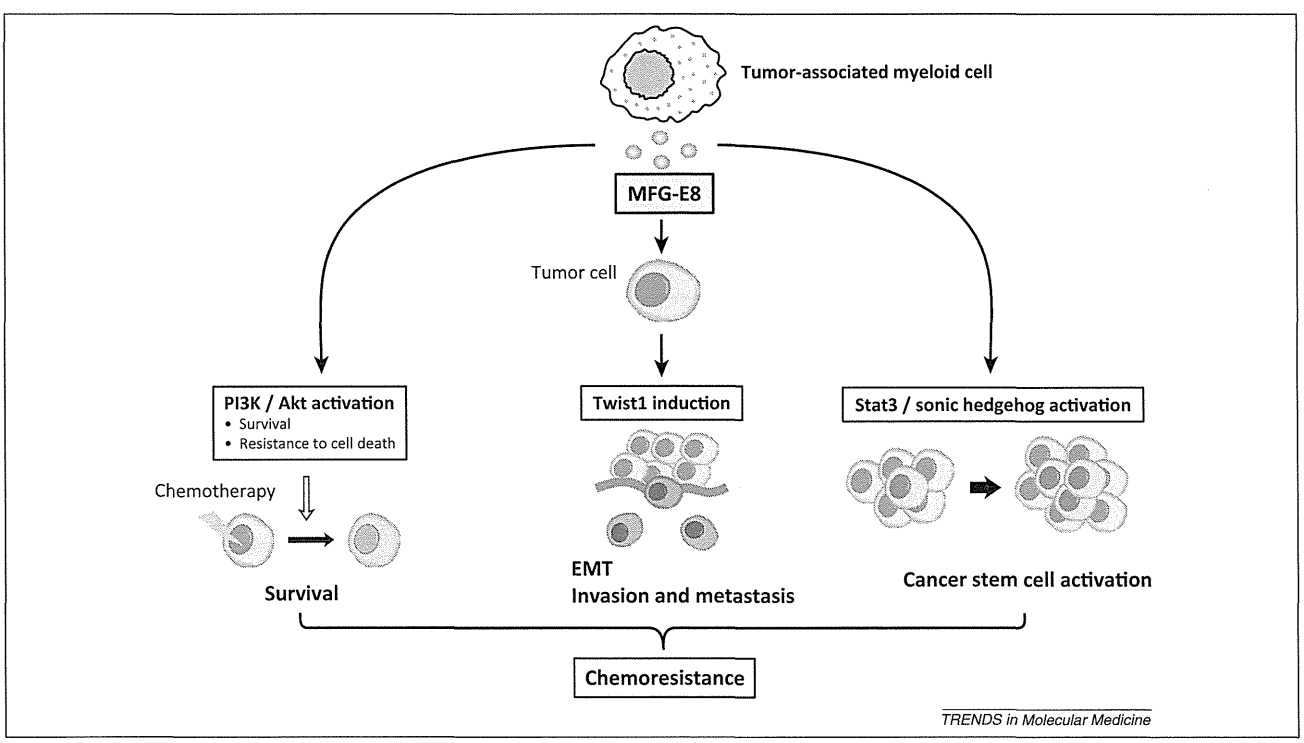

Figure 3. Myeloid cell derived MFG-E8 contributes to tumorigenic activities. MFG-E8, preferentially produced by tumor-associated myeloid cells, promotes tumorigenicity and suppresses anticancer drug responses by interfering with multiple nodes of oncogenic (PI3K–Akt and Stat3) and developmental (twist1) pathways and cancer stem cell activities (Stat3 and sonic Hedgehog). Abbreviations: MFG-E8, milk-fat globule-EGF factor VIII; PI3K, phosphatidylinositol-3-kinase; Stat, signal transducer and activator of transcription.

cells. Thus, therapeutic manipulation of the molecular pathways regulating tolerogenic phagocytosis would increase antitumor immunity upon treatment with conventional cytotoxic drugs that do not trigger ICD (Figure 4). Alternatively, each myeloid cell subset may have a distinct repertoire of phagocytic receptors, which determines tumorigenic activities and immune suppression. This assumption led us to hypothesize that MFG-E8 and Gas-6 may be preferentially recognized by immunosuppressive populations including M2-type macrophages, MDSCs, and Tie2-positive angiogenic monocytes, whereas CRT may prefer to interact with immunogenic M1-type macrophages and/or inflammatory monocyte-derived DCs. A comprehensive analysis regarding phagocytosis-mediated innate immune systems in tumor microenvironments would clarify their functional significance in the regulation of tumorigenicity and anticancer drug responses.

Regulation of chemotherapy-induced stress responses by myeloid cells: the mechanistic insight

Several types of chemotherapeutic agents have an intrinsic capacity to trigger ICD in mutated, hyperploid tumor cells, which coordinately programs innate immune systems to efficiently elicit antitumor immune responses. The induction of ICD is associated with the induction of ER stress, the generation of reactive oxygen species (ROS) and the activation of autophagy, leading to the cell surface translocation of CRT and the extracellular release of ATP [59,69,72]. Moreover, taxol or doxorubicin triggers mannose-6-phosphate expression on tumor cells, by which granzyme-B can infiltrate into tumor cells thus increasing susceptibility to chemotherapy [75]. Consequently, the intrinsic properties of tumor cells define the immunogenicity and immune-mediated regulation of chemosensitivity.

By contrast, most cytotoxic agents, such as cisplatin, dacarbazine, etc., do not trigger ER stress responses and thus do not evoke immunogenic antitumor responses. Moreover, an ICD inducer such as anthracyclin fails to induce antitumor immunogenicity in oncogene-derived spontaneous breast cancer models, suggesting that genetic or epigenetic profiles other than ER stress contribute to tumor immunogenicity [76]. In these conditions, genotoxic insults caused by chemotherapeutic agents facilitate the release of inflammatory mediators, which serve as drivers of protumorigenic inflammation and thus contribute to chemoresistance [2,45].

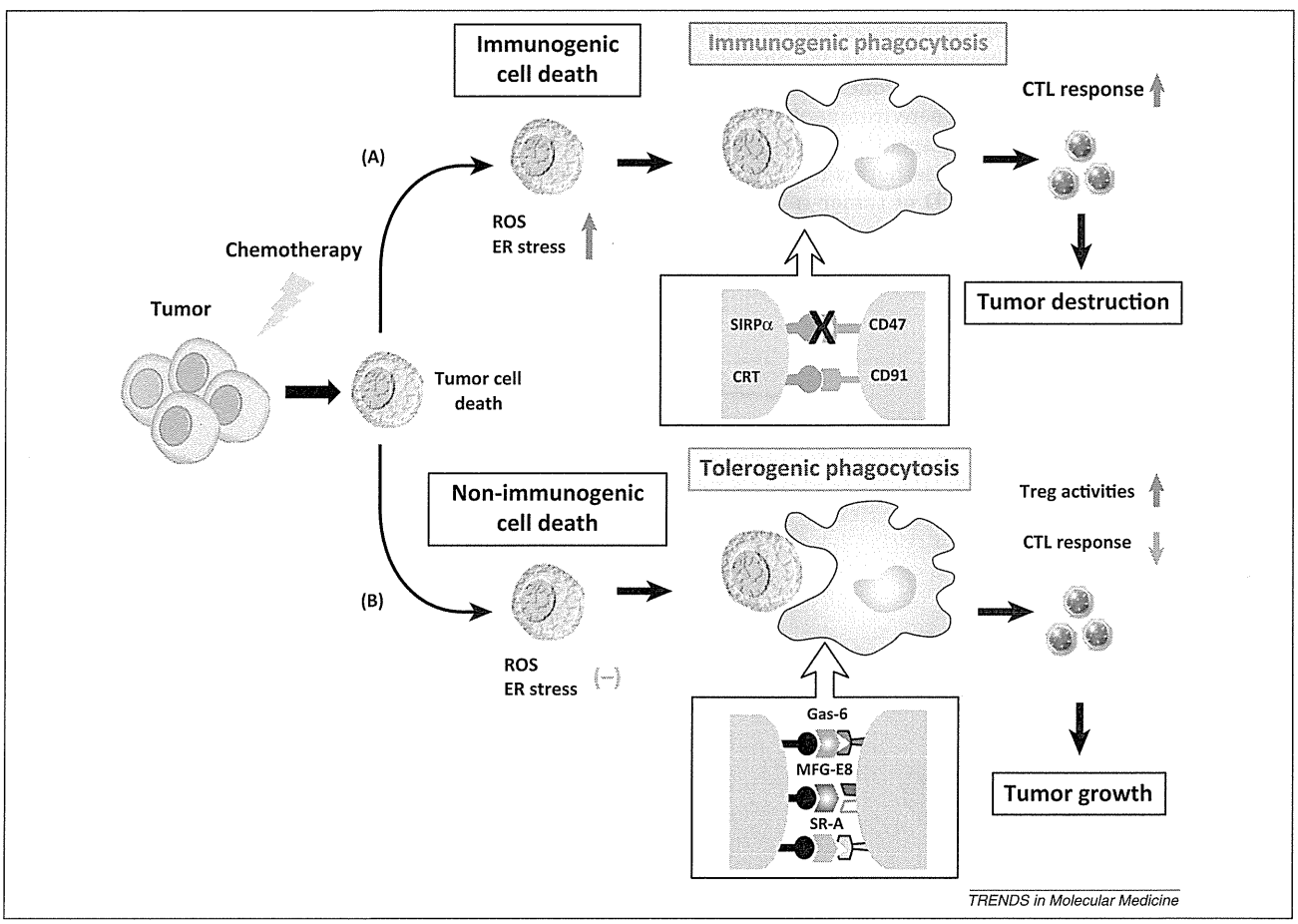

Figure 4. Phagocytic pathways regulate antitumor immunity and chemotherapeutic responses. Two modes of phagocytosis-mediated innate immune pathways are regulated by different classes of chemotherapeutic agents. **(A)** Some groups of cytotoxic agents, such as anthracycline, oxaliplatin, methotrexate, etc., increase immunogenicity of dying tumor cells (immunogenic cell death, ICD) and trigger immunogenic phagocytosis through the interaction between CRT and CD91, thus contributing to efficient processing and presentation of tumor-derived antigens to cytotoxic T lymphocytes. **(B)** By contrast, most chemotherapeutic agents do not induce tumor immunogenicity and thus result in non-immunogenic cell death. This initiates tolerogenic phagocytosis through MFG-E8, Gas-6, and SR-A-dependent mechanisms, leading to impaired antitumor immune responses. Abbreviations: CRT, calreticulin; MFG-E8, milk-fat globule-EGF factor VIII; SIRP, signal regulatory protein; ROS, reactive oxygen species.

Therefore, endogenous inflammatory signals triggered by anticancer therapies have two faces for regulating tumorigenic activities: they create an environment favoring induction of antitumor immunity in some cases, whereas they foster tumor-promoting inflammation in others. To counteract immunogenic environments created by chemotherapy-induced inflammation, tumor-associated myeloid cells may utilize multiple pathways to subvert the innate signals (such as the TIM-3–HMGB1 interaction) [20], as well as to promote tolerogenic phagocytosis (MFG-E8 and Gas-6) [63,66]. Moreover, myeloid cells may generate protumorigenic niches by manipulating PRR-mediated pathways (RAGE and TLR) [49,50,56]. In this regard, tumor microenvironments constitute a barrier system to protect them from excess inflammation induced by anticancer therapeutics through multiple pathways including antitumor innate immunity. At the same time, protumorigenic inflammatory signals remain intact due to the compensatory pathways that activate innate immune signals in myeloid cells. These regulatory systems that control the intensity and quality of therapy-induced acute inflammation may serve as an essential gatekeeper for avoiding overwhelming cellular damage that may be harmful to the tumor microenvironment integrity and maintaining protumorigenic smoldering inflammation (Figure 5).

Thus, targeting of negative regulators of innate immune pathways in myeloid cells may constitute a novel strategy to activate immune responses in coordination with acute inflammation triggered by cytotoxic chemotherapy, thus overcoming resistance to a broad range of 'non-immunogenic' anticancer regimens.

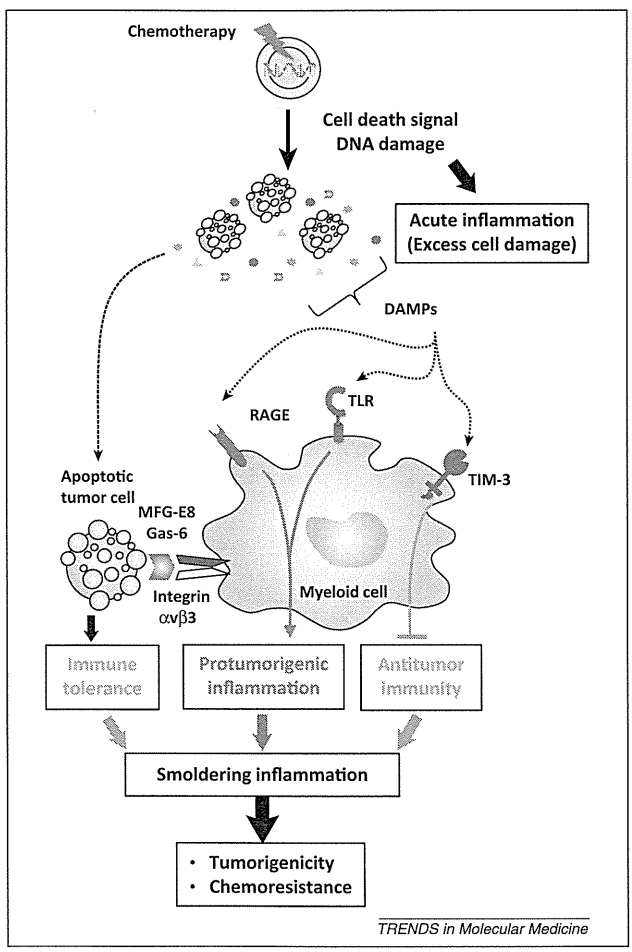

Figure 5. Myeloid cells protect tumors from chemotherapy-mediated excess inflammation. Tumor-associated myeloid cells control acute inflammation caused by anticancer therapeutics and thus may support tumor survival and create chemoresistant niches. Tumor-associated myeloid cells adopt multiple strategies to prevent excess chemotherapy-induced inflammation by manipulating DAMP-mediated innate immune signals and inducing tolerogenic phagocytosis. Conversely, certain types of inflammatory pathways mediated by TLR, RAGE, etc., remain unimpaired through compensatory machineries and serve as an output for protumorigenic inflammation. These regulatory systems play an essential role in controlling the intensity and quality of inflammation in tumor microenvironments, which maintain smoldering inflammatory signals and avoid acute cellular damage harmful to the integrity of tumor microenvironments. Abbreviations: DAMPs, damage-associated molecular patterns; TLR, Toll-like receptor; RAGE, receptor for advanced glycan end product; MFG-E8, milk-fat globule-EGF factor VIII; TIM-3, T cell immunoglobulin mucin domain protein-3.

Concluding remarks and future perspectives

We provide an overview of myeloid cell mediated regulation of anticancer therapies. Although ICD elicited by anticancer chemotherapy contributes to endogenous antitumor immunosurveillance, several negative regulatory pathways described here blunt the innate immune systems that can be manipulated to trigger antitumor immune responses by 'non-immunogenic' anticancer agents. Further elucidation of the role of myeloid cell derived negative regulators would greatly advance our knowledge about molecular linkages between myeloid cell mediated tumorigenicity and responses to anticancer therapeutics (Box 1).

Anticancer therapies confer selective pressure on surviving tumor cells to cause new genetic mutations and acquire drug resistance in support of the residual tumor microenvironment [1,3,77]. In the case of chemotherapeutic regimens that usually trigger ICD, genetic and epigenetic changes in the course of treatment may lead to mutations of genes or signal pathways related to ER stress responses. These alterations might suppress the extracellular release of DAMPs and ICD, causing 'non-immunogenic' cell death, tolerogenic phagocytosis, and chemoresistant tumor microenvironments. Thus, targeting the cell intrinsic machineries that suppress ER stress may be required to further improve the antitumor effect of 'immunogenic' chemotherapeutic agents. In this regard, the recent development of autophagy activators, which may stimulate downstream pathways of ER stress responses, may recover tumor immunogenicity and prevent the formation of chemoresistant niches by tumor-associated myeloid cells [78]. Further investigation is required to determine whether certain somatic mutations abate the ability of tumor cells to release DAMPs and if

Box 1. Outstanding questions

- The molecular mechanisms and upstream pathways responsible for creating protumorigenic and chemoresistant networks by myeloid cell derived soluble factors remain largely unknown.
- How PRR-mediated recognition of DAMPs manipulates myeloid cells to support protumorigenic inflammatory signals but repress antitumor innate immune responses in tumor microenvironments remains to be determined.
- How diverse sets of phagocytic receptors mediate immunogenic or tolerogenic immunity upon encounter of apoptotic tumor cells remains unknown.

therapeutic manipulation of these pathways increases immunogenicity and inflammation in tumor microenvironments.

It is clear that complex networks composed of non-transformed cells have a tremendous impact on the prognosis and treatment outcomes of advanced cancer [3,69]. The identification of cellular and molecular pathways that participate in the interaction between tumorigenic cells and myeloid cells provides an invaluable opportunity for therapeutic interventions that may concomitantly overcome chemoresistant niches and translate our understanding of cancer-related inflammation into meaningful therapeutic advances.

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Immunity
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TIM-4 Glycoprotein-Mediated Degradation of Dying Tumor Cells by Autophagy Leads to Reduced Antigen Presentation and Increased Immune Tolerance

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SUMMARY

Phagocytosis of apoptotic cells by myeloid cells has been implicated in the maintenance of immune homeostasis. In this study, we found that T cell immunoglobulin- and mucin domain-containing molecule-4 (TIM-4) repressed tumor-specific immunity triggered by chemotherapy-induced tumor cell death. TIM-4 was found to be highly expressed on tumor-associated myeloid cells such as macrophages (TAMs) and dendritic cells (TADCs) and danger-associated molecular patterns (DAMPs) released from chemotherapy-damaged tumor cells induced TIM-4 on tumor-associated myeloid cells recruited from bone marrow-derived precursors. TIM-4 directly interacted with AMPKα1 and activated autophagy-mediated degradation of ingested tumors, leading to reduced antigen presentation and impaired CTL responses. Consistently, blockade of the TIM-4-AMPKα1-autophagy pathway augmented the antitumor effect of chemotherapeutics by enhancing tumor-specific CTL responses. Our finding provides insight into the immune tolerance mediated by phagocytosis of dying cells, and targeting of the TIM-4-AMPKα1 interaction constitutes a unique strategy for augmenting antitumor immunity and improving cancer chemotherapy.

INTRODUCTION

Tumor microenvironments play a determinant role in suppressing responsiveness to anticancer drugs and accelerating subsequent tumor growth (Hanahan and Coussens, 2012). Therefore, it is extremely difficult to treat established tumors in which the complex interplay between tumor cells and stromal components is already skewed in favor of tumor progression. Recent evidences have clarified the critical role of intrinsic host immunity in the regulation of tumor sensitivities to anticancer drugs. In addition, the interactions between antigen-presenting cells (APCs) and T cells, which are regulated by various repertories of costimulatory and coinhibitory molecules in tumor microenvironments, might impact tumor progression and anticancer drug resistance (Peggs et al., 2007). Consistent with this notion, antibody blockade of the major coinhibitory molecules cytotoxic T lymphocyte antigen-4 (CTLA-4) or programmed death-1 (PD-1) enhances antitumor responses in patients who have been refractory to other anticancer modalities (Pardoll, 2012). Thus, the molecular events governing host immunoregulatory elements might modulate responsiveness to conventional anticancer treatments.

T cell immunoglobulin and mucin domain protein-4 (TIM-4), which is expressed on myeloid cells including dendritic cells (DCs) and macrophages from spleen, lymph nodes, or peritoneal cavity, serves as a critical sensor for controlling the functions of naive and activated T cells. TIM-4 differentially regulates T cell homeostasis by inhibiting naive T cells during the induction phase of an immune response and enhancing T cell responses at the effector phase (Rodriguez-Manzanet et al., 2010). In addition, TIM-4 plays a critical role in phagocytosis of apoptotic cells by APCs through interaction with phosphatidylserine (PS) (Miyanishi et al., 2007; Kobayashi et al., 2007). Although these studies verify the role of TIM-4 in the regulation of immune homeostasis by controlling APC functions, it remains unknown how TIM-4 impacts host immunity within tumor microenvironments. In particular, it is critical to evaluate the role of TIM-4 expressed on myeloid cells of distinct tissues including tumors in regulating immunity and tolerance.

In this study, we identified a new pathway whereby TIM-4-adenosine monophosphate activating kinase-α1 (AMPKα1) interaction in myeloid cells triggers antigen-specific immune tolerance by inducing autophagy-mediated degradation of chemotherapy-killed apoptotic tumor cells. Thus, targeting TIM-4-AMPKα1 interplay might constitute a novel strategy for potentiating the antitumor responses.

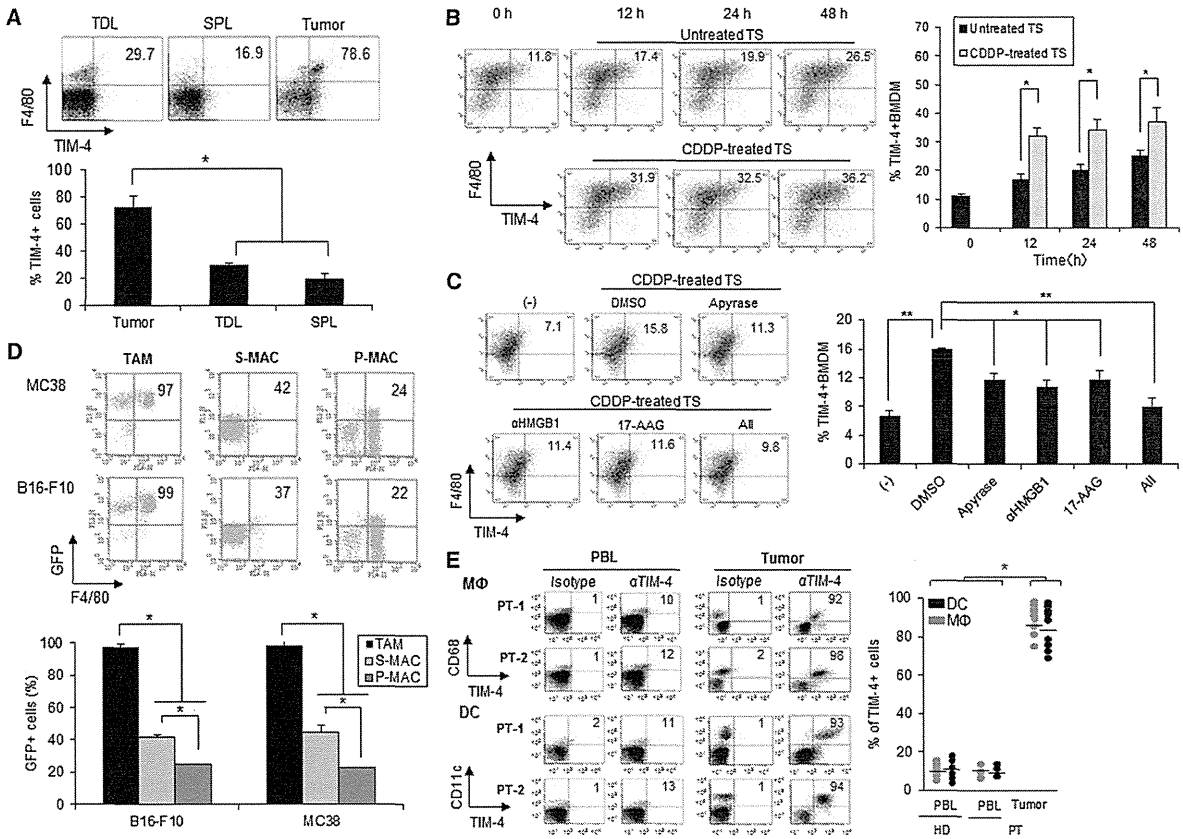

Figure 1. Tumor-Derived DAMPs Upregulate TIM-4 on Myeloid Cells

(A) Expression of TIM-4 on F4/80$^+$ macrophages from tumors, TDL, or SPL of B16-F10 tumor-bearing mice.

(B) TIM-4 on BMDMs treated with the tumor supernatants (TS) of CDDP-treated B16-F10 cells.

(C) TIM-4 on BMDMs cultured with CDDP-treated TS pretreated with blocking Ab for αHMGB1, 17-AAG, Apyrase or all.

(D) GFP$^+$ proportions in F4/80$^+$ TAMs, S-MAC, or P-MAC in lethally irradiated WT C57BL/6 mice transplanted with bone marrow cells from GFP-transgenic mice.

(E) TIM-4 on macrophages in PBL of HD (n = 6) or cancer patients (PT) (n = 3), TAM or TADCs from the same patients (n = 13). Data are shown as mean ± SD (n = 3–5). See also Figure S1.

RESULTS

DAMPs Released from Chemotherapy-Treated Tumor Cells Upregulate TIM-4 on Tumor-Associated Macrophages and Dendritic Cells

We first evaluated TIM-4 expression on myeloid cells from mice bearing established tumors. Single-cell suspensions prepared from subcutaneous B16-F10 tumors revealed high percentages of TIM-4-expressing CD11b$^+$F4/80$^+$ macrophages and CD11c$^+$ MHC-II^{hi} dendritic cells (DCs) (Figure 1A; Figure S1B). In contrast, lower percentages of TIM-4$^+$ macrophages were found in the tumor-draining lymph nodes (TDL) and spleens (SPL) of tumor-bearing mice (Figure 1A). TIM-4 was not detectable on lymphocytes (T, B, and NK cells), EpCAM$^+$ epithelial cells, CD31$^+$ endothelial cells, or CD45$^-$ gp38$^+$ stromal cells in tumor tissues and spleens of tumor-bearing mice, suggesting that TIM-4 expression was confined to myeloid lineage cells (Figure S1A; not shown).

To investigate tumor-derived factors contributing to TIM-4 induction in myeloid cells, we incubated immature bone-marrow-derived macrophages (BMDMs) or dendritic cells (BMDCs) with untreated or cisplatin (CDDP)-treated B16-F10 tumor cell supernatants. TIM-4 expression was upregulated 12 hr after treatment with the supernatants of CDDP-treated tumor cells at greater levels than those of untreated cells (Figure 1B). Moreover, TIM-4 was also induced by the supernatants of MC38 colon cancer cells treated with CDDP, camptothecin (CPT-11), or gemcitabine (GEM), as well as 3LL lung cancer cells treated with CDDP, taxol, or GEM, compared to the cells treated with DMSO (Figure S1C), suggesting that soluble factors released from chemotherapy-treated, dying tumor cells contribute to the TIM-4 upregulation on tumor-associated myeloid cells. Dying tumor cells release endogenous ligands for pattern recognition receptors referred as damage-associated molecular patterns (DAMPs) (Krysko et al., 2012). Although treatment of the dying tumor cell supernatant with an inhibitor for high mobility group box-1 (HMGB1) (anti-HMGB1 Ab), heat shock protein 90 (HSP90) (17-AAG), or ATP (apyrase) singly had only marginal inhibitory effects on TIM-4 expression on BMDMs, a combination of all three inhibitors largely diminished the upregulation of TIM-4 on BMDMs

(Figure 1C). Conversely, treatment with HMGB1, ATP, monosodium urate (MSU), and S100A8 together caused TIM-4 upregulation on BMDMs or BMDCs (Figure S1D and S1E). Thus, an inflammatory tumor microenvironment triggered by cytotoxic chemotherapy induces the TIM-4 upregulation on tumor-associated macrophages and DCs through the release of DAMPs.

To determine the origin of TIM-4$^+$ TAMs, we examined TAMs from various tumors established in C57BL/6 mice transplanted with bone marrow cells (BMCs) from GFP-transgenic mice (Figure 1D). As previously reported (Geissmann et al., 2010), peritoneal macrophages were largely radioresistant and contained about 20% of BMC-derived GFP$^+$ cells, and the GFP$^+$F4/80$^+$ cells were detected in spleens at higher frequency than peritoneal cavity. In contrast, the majority of TAMs were bone-marrow-derived GFP$^+$ cells (Figure 1D). Although previous reports revealed that splenic inflammatory CCR2^{hi} monocytes might serve as a main reservoir of TAMs (Qian et al., 2011; Cortez-Retamozo et al., 2012), these results suggest that majority of TAMs might be originated from BMCs.

Finally, we found that TIM-4 expression was also detected on higher percentages of TAM or TADCs isolated from tumor tissues of patients with advanced non-small-cell lung or gastric carcinoma than on the same populations obtained from peripheral blood leukocytes (PBL) of cancer patients or healthy volunteers (HV) (Figure 1E). These results demonstrate that tumor-derived DAMPs are responsible for upregulating TIM-4 on myeloid cells.

TIM-4 Differently Regulates Antigen Presentation in Distinct Macrophage Subsets

Several lines of evidence have revealed the impact of apoptotic cell engulfment on maintaining immune tolerance and preventing excess inflammation (Nagata et al., 2010; Griffith and Ferguson, 2011). Although TIM-4 serves as a PS receptor that facilitates phagocytosis of apoptotic cells by peritoneal macrophages (P-MAC), the role of TIM-4 in regulation of phagocytosis by tumor-infiltrating myeloid cells remains unknown.

To address this issue, we prepared for B16-F10 TAMs or bulk P-MAC of C57/Bl6 wild-type (WT) mice, which highly expressed TIM-4 (data not shown), as well as those of TIM-4-deficient mice. In addition, we isolated TIM-4$^+$ and TIM-4$^-$ populations of BMDMs or splenic macrophages (S-MAC) from WT C57BL/6 mice or those from TIM-4-deficient (*Timd4*$^{-/-}$) mice. We confirmed that TIM-4 expression was not detected in TIM-4$^-$ WT cells even after ingestion of dying tumor cells in our experimental conditions (data not shown). In TIM-4$^+$ WT TAMs, BMDMs, or splenic macrophages, we found that TIM-4 was dispensable for phagocytosis of dying EG7 tumor cells, because the phagocytosis by these cells was not different in TAMs or BMDMs of TIM-4$^+$ or TIM-4$^-$ WT and *Timd4*$^{-/-}$mice, and remained unaffected by treatment with anti-TIM-4 mAb (Figures 2A and 2B; Figures S2A–S2C). In contrast, phagocytosis of apoptotic EG7 cells was significantly inhibited in *Timd4*$^{-/-}$ and WT peritoneal macrophages treated with the TIM-4 mAb or a TIM-4-specific siRNA that suppressed TIM-4 expression (siRNA #2) (Figures S1D, S2B–S2E). The blocking activities for TIM-4 were intact even when Fab fragments were used, and they were not affected by Fc receptor blocking with anti-CD32/CD16 mAb (data not shown). These results suggest differential contribution of TIM-4 to phagocytosis of apoptotic cells by distinct subsets of macrophages, which might be determined by expression of other phagocytic receptors.

We next analyzed how TIM-4 regulates antigen presentation by TAMs and TADCs. TAMs and TADCs were isolated from B16-OVA-bearing WT or *Timd4*$^{-/-}$ mice treated with or without CDDP and then used for antigen presentation (SIINFEKL-H2K^b expression). We found that both TAMs and TADCs from *Timd4*$^{-/-}$ mice had a superior ability to form SIINFEKL-H2K^b complex from OVA-expressing B16 tumors pretreated with CDDP compared to those from WT counterparts (Figure 2C). Moreover, TIM-4$^+$ BMDMs treated with anti-TIM-4 mAb augmented cross-priming of OVA-specific T cells at similar levels to *Timd4*$^{-/-}$ BMDMs (Figures S2A and S2C). Interestingly, while treatment with anti-TIM-4 mAb or siRNA augmented SIINFEKL-H2K^b presentation by TIM-4$^+$ TAMs (Figures S2B and S2E), BMDMs, or splenic macrophages (Figure S2H), the antigen presentation was substantially impaired in *Timd4*$^{-/-}$ TAMs or WT TAMs treated with anti-TIM-4 mAb, likely due to the impaired phagocytosis (Figure 2C; Figures S2C and S2E).

To examine whether TIM-4 regulates APC-mediated cross-priming of tumor antigen-specific CD8$^+$ cytotoxic T lymphocytes (CTLs), TAMs and TADCs were isolated from B16-OVA-bearing WT or *Timd4*$^{-/-}$ mice treated with or without CDDP and then used for cross-priming of OVA-specific OT-I cells. TIM-4 deficiency augmented interferon-γ (IFN-γ) production in TCR-Vβ5$^+$ OT-I cells when stimulated by TAMs and TADCs isolated from CDDP-treated B16-OVA tumors, although cross-priming ability of TADCs were superior to that of TAMs (Figure 2D). Moreover, OVA-specific responses to TIM-4$^+$ BMDMs were also augmented by anti-TIM-4 mAb when B16-OVA cells were loaded after treatment other cytotoxic agents such as CPT-11, taxol, or GEM (Figure S2F).

The blockade of TIM-4 augmented antigen cross-presentation while ingestion of dying tumor cells by TAMs remained intact, suggesting that some other phagocytic receptors might compensate and stimulate antigen processing by TAMs. Because recent studies demonstrated the importance of CD91-calreticulin (CRT) interaction in stimulating phagocytosis and cross-presentation of immunogenic antigens by APCs (Obeid et al., 2007), we examined whether CD91 was utilized as an alternative receptor for phagocytosis by TAMs treated with anti-TIM-4 mAb. Although CD91 was expressed on TAMs at comparable levels to P-MAC, treatment with anti-CD91 neutralizing Ab and anti-TIM-4 mAb significantly suppressed the ingestion of dying EG7 cells and antigen-presentation by TAMs (Figure S2G). Thus, multiple phagocytic receptors allow TAMs to utilize TIM-4-independent compensatory pathways.

In process of analyzing the fate of engulfed tumor cells in TAMs, we found that the ingested apoptotic tumor cells were located into LAMP1$^+$ lysosomal compartment in TIM-4$^+$ BMDMs at much higher frequencies than those in *Timd4*$^{-/-}$ BMDMs (Figure 2E). This finding led us to hypothesize that TIM-4 might promote lysosomal degradation of the ingested tumor cells, leading to the impaired tumor antigen presentation by TIM-4$^+$ TAMs. Consistent with this notion, a lysosomal acidification inhibitor bafilomycin A1 increased the ability of TIM-4$^+$ BMDMs to stimulate OT-I cells to a comparable level with that of TIM-4$^-$ BMDMs (Figure S3A).

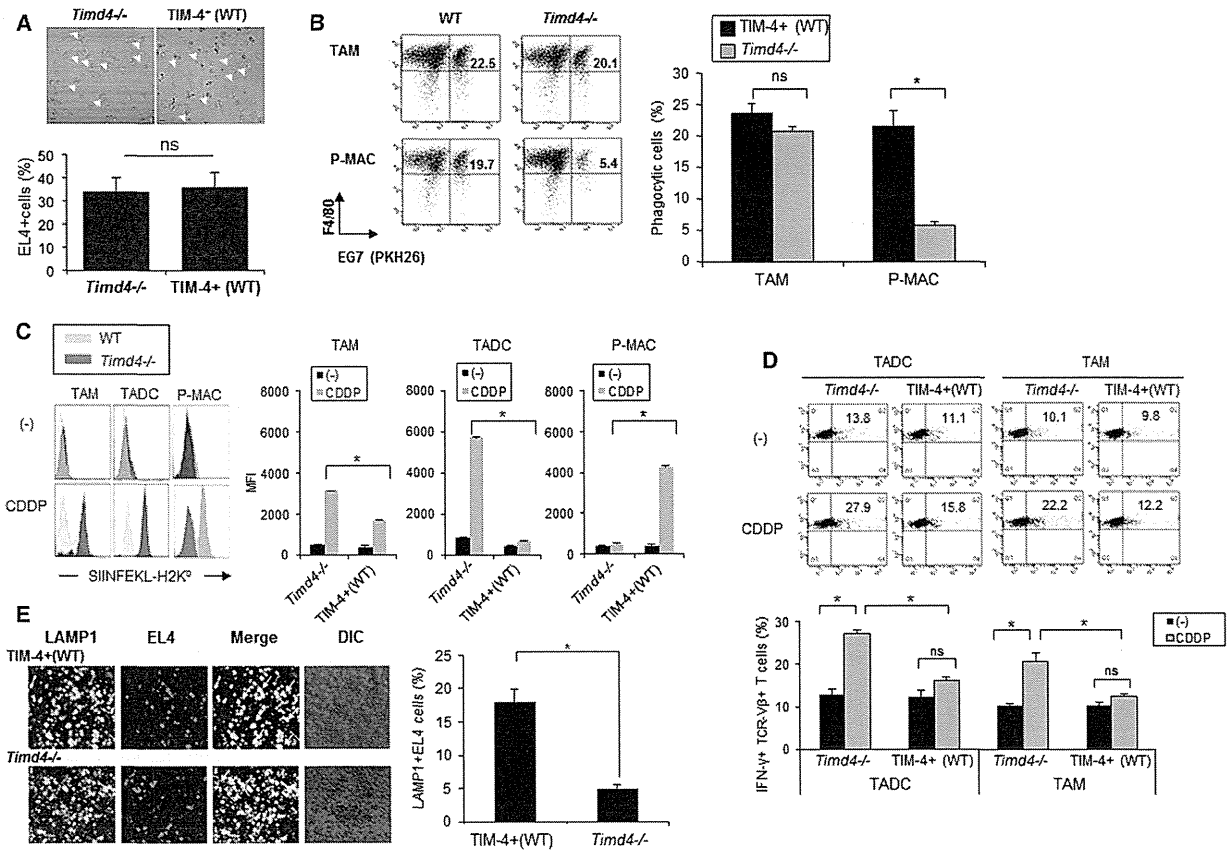

Figure 2. TIM-4 Suppresses APC Activities by Dying Tumor Cells

(A) Ingestion of TIM-4$^+$ WT or *Timd4*$^{-/-}$ BMDMs loaded with CDDP-treated PKH26$^+$EG7 cells.

(B) Ingestion of TAMs and P-MAC from WT or TIM-4$^{-/-}$ mice with CDDP-treated EG7 cells.

(C) OVA antigen presentation of TAMs, TADCs, or P-MAC isolated from tumors of *Timd4*$^{-/-}$ mice inoculated with OVA-B16 cells. The MFI of SIINFEKL-H2K^{b+} was quantified by flow cytometry.

(D) Percentage of IFN-γ$^+$ cells in OT-1 cells cocultured with TADCs or TAMs.

(E) Lysosomal degradation of TIM-4$^+$ WT or *Timd4*$^{-/-}$ BMDMs loaded with CDDP-treated CFSE$^+$ EL4 cells. Data are shown as mean ± SD (n = 3–5). See also Figure S2.

Together, these results suggest that TIM-4 contributes to immune tolerance by promoting phagosomal acidification and antigen overdegradation in macrophages localized in tumors, bone marrow, or spleen.

TIM-4 Suppresses Antigen Presentation by Autophagy-Mediated Mechanisms

We next examined which signaling cascades are involved in the impaired antigen presentation by TIM-4$^+$ BMDMs. Since treatment with an mammalian target of rapamycin (mTOR) inhibitor rapamycin, a phosphoinositide 3-kinase (PI3K) class-IA p110-δ-specific inhibitor IC87114 or the genetic deficiency of p85α repressed OT-I cell activation by TIM-4$^-$ BMDMs to comparable levels with TIM-4$^+$ counterparts. In contrast, the treatment with a PI3K class III-specific inhibitor 3-methyladenine (3-MA) augmented the activation by TIM-4$^+$ BMDMs to the levels comparable with TIM-4$^-$ counterparts (Figures S3A and S3B).

These findings led us to hypothesize e involvement of autophagy on TIM-4-mediated immune regulation, because autophagy serves as a main pathway regulated by multiple signaling cascades such as PI3K and mTOR (Levine et al., 2011). Moreover, autophagy is critically involved in multiple processes of immune regulation, such as MHC class II-mediated antigen presentation and regulation of cytokine profiles responses (Lee et al., 2010; Saitoh et al., 2008). Furthermore, the TIM-4-mediated uptake of apoptotic cells facilitated microtubule-associated protein light chain 3 (LC3)-associated phagosome formation (Martinez et al., 2011). Thus, we examined whether TIM-4 might promote degradation of ingested cells by activating autophagic pathways. Consistent with this idea, ingestion of dying EL4 cells resulted in enhanced autophagic activation of TIM-4$^+$ BMDMs generated from LC3-GFP transgenic mice (Mizushima et al., 2004), as shown by the increased frequencies of punctuate form of LC3 (Figure 3A). In addition, phagocytosis of dying EG7 cells decreased intensity of LC3 levels in TIM-4$^+$ WT BMDMs, which suggest increased autophagy-mediated degradation, whereas LC3 expression remained unchanged in *Timd4*$^{-/-}$ BMDMs after engulfment of dying EG7 cells

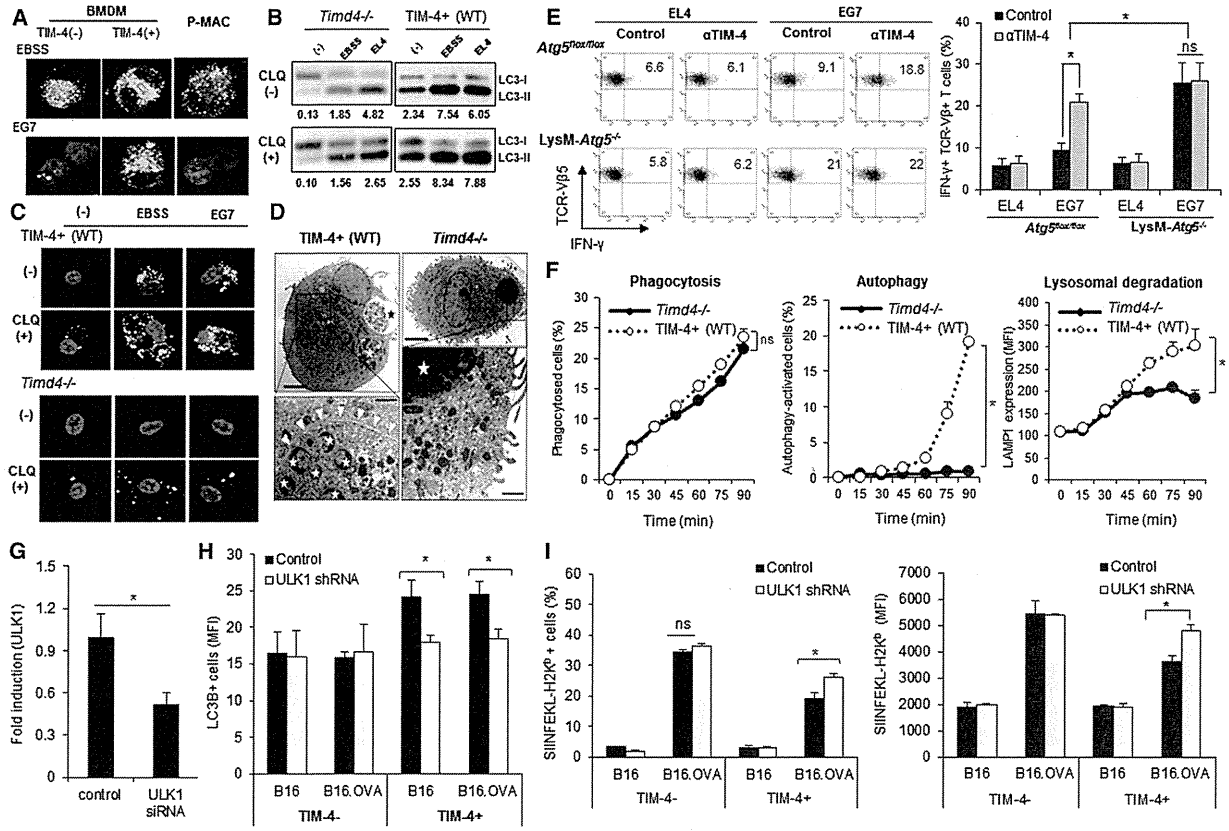

Figure 3. TIM-4 Regulates Cross-Presentation by Activating Autophagic Pathways

(A) Distribution of LC3 in TIM-4$^+$, TIM-4$^-$ BMDMs, or P-MAC from LC3-GFP-TG mice loaded with CDDP-treated EG7 cells or received amino-acid starvation (EBSS).

(B and C) LC3-I/II levels in TIM-4$^+$ or *Timd4*$^{-/-}$ BMDMs loaded with CDDP-treated EG7 cells or received EBSS with or without CLQ.

(C) Distribution of LC3 in TIM-4$^+$ or *Timd4*$^{-/-}$ BMDMs treated as shown in (B).

(D) Formation of autophagosome in TIM-4$^+$ or *Timd4*$^{-/-}$ BMDMs as shown in (B). The autophagic vesicles (arrow head) containing dying cell debris (*) were shown. Scale bar represents 4 μm (upper panel) and 1 μm (lower panel).

(E) IFN-γ production in OT-I cells cocultured with BMDMs from WT or LysM-*Atg5*$^{-/-}$ mice loaded with CDDP-treated EL4 or EG7 cells and control Ig or αTIM-4.

(F) Phagocytosis, autophagy, and lysosomal degradation of TIM-4$^+$ WT or *Timd4*$^{-/-}$ BMDMs loaded with CDDP-treated PKH26$^+$EL4 cells for the indicated time. The MFI of PKH26, LAMP1, or LC3B levels in the F4/80$^+$ cells were evaluated by flow cytometry. The phagocytosed and autophagy-activated cells were calculated as increased percentages of PKH26$^+$ EL4 cells and reduced percentages of LC3-positive cells loaded with dying EL4 cells compared to untreated cells, respectively.

(G) Efficiency of ULK1 inhibition in BMDMs transfected with ULK1 siRNA.

(H and I) LC3B levels (H) or the presentation of SIIINFEKL-H2K^b (I) in ULK1 siRNA-transfected TIM-4$^+$ or TIM-4$^-$ BMDMs loaded with CDDP-treated B16 or B16-OVA cells. The MFI of SIIINFEKL-H2K^{b+} cells are shown. Data are shown as mean ± SD (n = 3–5). See also Figure S3.

(Figure S3C). TIM-4$^+$ WT BMDMs increased autophagic flux as shown by enhanced LC3-II with lysosomal inhibitor chloroquine (CLQ), further confirming the role of TIM-4 in stimulating autophagic response, whereas autophagic flux was not observed in TIM-4$^{-/-}$ BMDMs upon ingestion of apoptotic cells (Figures 3B and 3C). Electron microscopic analysis further revealed that the formation of autophagosome with double-layer membranes containing degrading cell debris and organelles was detected in TIM-4$^+$ BMDMs, but autophagic vesicles were barely detectable in *Timd4*$^{-/-}$ BMDMs, upon engulfment of dying cells (Figure 3D).

To further gain insights into the importance of autophagic machinery in the TIM-4- mediated regulation, we generated LysM-Cre × *Atg5*$^{flox/flox}$ conditional gene-targeted (LysM-*Atg5*$^{-/-}$) mice, which are specifically defective of autophagy in myeloid lineage cells. We confirmed that Atg5 was specifically deleted and autophagic activities were impaired in the LysM-*Atg5*$^{-/-}$ BMDMs (data not shown). F4/80$^+$CD11b$^+$ macrophages were generated from bone marrow cells of WT or LysM-*Atg5*$^{-/-}$ mice, loaded with dying EL4 or EG7 cells for 4 hr, and then cocultured with OVA-specific OT-I cells. LysM-*Atg5*$^{-/-}$ BMDMs had a greater potential in stimulating OT-I cells as compared to WT BMDMs, and the levels of antigen presentation by LysM-*Atg5*$^{-/-}$ BMDMs were comparable to those by TIM-4$^{-/-}$ BMDMs (Figure 3E). In accord with the significance of crosstalk between TIM-4 and autophagic pathways, treatment with anti-TIM-4 mAb or siRNA-mediated inhibition of TIM-4 had little

additional effects on the antigen presentation or the cross-priming of OT-I cells by TIM-4$^+$LysM-*Atg5*$^{-/-}$ BMDMs (Figure 3E; Figure S3D). Furthermore, whereas the anti-TIM-4 mAb-mediated enhancement of cross-priming by WT BMDMs was abolished by rapamycin and IC87114, the enhanced cross-priming by LysM-*Atg5*$^{-/-}$ BMDMs was not affected by these inhibitors (Figure S3E).

The LAMP1$^+$ phagolysosome containing dying cells was detected in TIM-4$^+$ BMDMs at similar levels to *Timd4*$^{-/-}$ BMDMs up to 30 min after phagocytosis of dying EL4 cells. However, the LAMP1 levels increased continuously in TIM-4$^+$ BMDMs, while it gradually decreased in *Timd4*$^{-/-}$ BMDMs after 30 min, whereas the kinetics of phagocytosis were similar in TIM-4$^+$ WT and *Timd4*$^{-/-}$ BMDMs (Figure 3F). Furthermore, autophagy was activated in TIM-4$^+$ BMDMs but not *Timd4*$^{-/-}$ BMDMs after ingestion of dying EL4 cells, suggesting that autophagy activation occurred selectively in TIM-4$^+$ cells (Figure 3F). These results suggested that TIM-4 was dispensable for phagolysosome formation following apoptotic cell phagocytosis, but it played a critical role in initiating autolysosome formation, leading to excessive antigen degradation and reduced cross-presentation by TIM-4$^+$ macrophages.

Recent studies have revealed that engulfment of TLR-associated particles or recognition of PS mediates a recruitment of LC3 to mature phagosomes through class III PI3K and Atg5-dependent but Unc51-like linase-1 (ULK1)-independent machineries (Sanjuan et al., 2007; Martinez et al., 2011). This process, referred as LC3-associated phagocytosis (LAP), does not cause an accumulation of autophagic vesicles and is responsible for facilitating dying cell clearance and TLR-mediated innate immune responses (Martinez et al., 2011; Henault et al., 2012). However, we observed the clear autophagic vesicles, which were colocalized with luminal ER tracker in TIM-4$^+$ BMDMs upon tumor cell phagocytosis (Figure 3A; data not shown). Moreover, siRNA-mediated targeting of ULK1, which efficiently inhibits ULK1 expression (Figure 3G), reversed the TIM-4-mediated activation of autophagy and the repressed cross-presentation in TIM-4$^+$ BMDMs upon ingestion of EL4 cells (Figures 3H and 3I). Thus, our findings strongly suggest that TIM-4 induces tolerogenic properties of myeloid cells upon recognition of apoptotic cells in conventional autophagy-dependent but LAP-independent manner. Then, we further investigated how TIM-4 regulates conventional autophagy after engulfment of dying tumor cells.

TIM-4 Interacts with AMPKα1 in Phagosome and Activates Autophagic Processes

Autophagy is tightly regulated by multiple molecules and pathways sensing stresses in the cells (Levine et al., 2011). Interestingly, TIM-4 was expressed mostly on the cell surface, but it was localized at LAMP1$^+$ phagolysosome after ingestion of apoptotic EL4 cells (Figure S4A), suggesting that TIM-4 might act on phagosome to regulate autophagy. We next tried to identify the molecule that directly interacts with TIM-4 and regulates autophagy. Although the autophagic activity is controlled by mTORC1 pathway regulators, such as SH3BP1, REDD, GSK-β, and FoxO (Levine et al., 2011), we failed to detect the involvement of these factors in the TIM-4-mediated regulation of autophagy (data not shown).

Recent studies have revealed that AMPKα interacts with ULK1 and activates autophagy (Egan et al., 2011; Kim et al., 2011). Furthermore, AMPKα was detected in macrophages upon the phagocytosis of apoptotic cells (Bae et al., 2011), which stimulated autophagy by inhibiting tuberous sclerosis protein-2 (TSC2)-mTORC1 (mTOR complex 1) signals (Hardie, 2007). Thus, we investigated whether AMPKα was involved in the TIM-4-mediated regulation of autophagy. We found that TIM-4 interacted with AMPKα1 in TIM-4$^+$ BMDMs after ingestion of dying EL4 cells, although TIM-4 binding with AMPKα1 was detected at lower levels even without phagocytic process (Figure 4A). We next examined the impact of TIM-4 on the activation of AMPK and autophagy. Phosphorylation of AMPKα1 at Thr172, which is critical for its kinase activities, was clearly induced in TIM-4$^+$ BMDMs, but not in *Timd4*$^{-/-}$ BMDMs or P-MACs, upon ingestion of dying EL4 cells (Figure 4B). More interestingly, TIM-4 was colocalized with AMPKα1 in LAMP1$^+$ and LC3$^+$ autophagolysosomal vesicles after phagocytosis of apoptotic cells (Figures 4C and 4D).

We next examined the role of the TIM-4-AMPKα interaction in the regulation of mTOR signals and autophagic activities. The inhibition of AMPKα in TIM-4$^+$ BMDMs augmented mTORC1 activities as shown by the increased phosphorylation of p70 and p85 S6K1 (Figure S4B). Moreover, TIM-4-AMPKα interaction promoted phosphorylation of ULK1 at Ser555, which is critical to recruit Atg13/FIP200 and initiate autophagic vesicle formation (Egan et al., 2011) (Figure 4E). TIM-4 and AMPKα1 were colocalized with ULK1 in LAMP1$^+$ lysosomal vesicles (Figure 4F). The importance of TIM-4-AMPKα1 interaction in the regulation of autophagy was further validated by analyzing AMPKα1-deficient (*Prkaa1*$^{-/-}$) TIM-4$^+$ BMDMs, because LC3-II expression was impaired in *Prkaa1*$^{-/-}$ BMDMs upon ingestion of EL4 cells (Figure 4G; Figure S5A). In contrast, AMPKα2 deficient (*Prkaa2*$^{-/-}$) TIM-4$^+$ BMDMs activated autophagy upon ingestion of dying EL4 cells at similar levels to WT counterparts, indicating that α1-subset of AMPK specifically interacts with TIM-4. These results are consistent with previous reports that AMPKα1 is a sole subset specifically expressed in myeloid cells (Sag et al., 2008; Yang et al., 2010). Moreover, BMDCs activated autophagy upon ingestion of dying tumor cells by TIM-4-dependent mechanisms, suggesting that TIM-4-mediated activation of autophagy is commonly operated in macrophages and DCs (data not shown).

The above finding led us to hypothesize that different dynamism and/or activities of AMPKα1 might be a determinant in regulating the APC activities of TIM-4$^+$ macrophages. The phosphorylation levels of AMPKα1 were comparable among TAMs, BMDMs, and peritoneal macrophages upon ingestion of apoptotic cells (data not shown). However, TIM-4 was colocalized with AMPKα1 in the intracellular compartments of TAMs and TIM-4$^+$ BMDMs upon ingestion of apoptotic cells, whereas TIM-4 was mostly retained on plasma membrane and thus scarcely interacted with AMPKα1 in peritoneal macrophages (Figure 4H). These results suggest the existence of molecular machineries regulating TIM-4 internalization after recognition of apoptotic cells, which differed among macrophage subsets.

Collectively, TIM-4 activates AMPKα1 in TAMs and BMDMs upon ingestion of apoptotic cells, leading to the ULK1-mediated formation of autophagic vesicles.

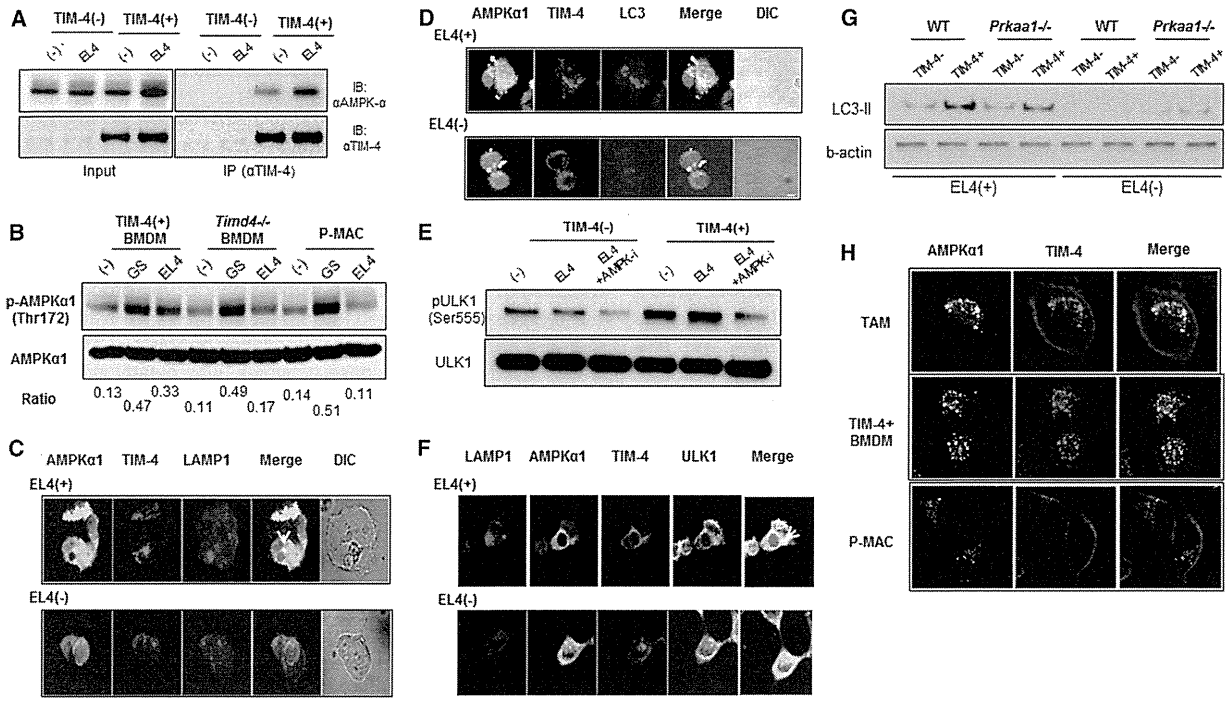

Figure 4. TIM-4-AMPKα1 Interaction Is Critical for Activating Autophagy

(A) CoIP of TIM-4 with endogenous AMPK-α in TIM-4$^+$ BMDMs loaded with CDDP-treated EL4 cells.

(B) Phosphorylated and total levels of AMPKα1 in TIM-4$^+$, *Timd4*$^{-/-}$ BMDMs, or P-MAC loaded with CDDP-treated EL4 cells. The ratio of p-AMPKα1/total AMPKα1 densitometry was shown.

(C and D) Localizations of TIM-4, AMPKα1, and LAMP1$^+$ (C) or LC3 (D) in TIM-4$^+$ BMDMs cultured with or without CDDP-treated EL4 cells. (E) Phosphorylation of ULK1 in TIM-4$^+$ or TIM-4$^-$ WT BMDMs were treated as described in (B).

(F) Localizations of LAMP1, TIM-4, AMPKα1, and ULK1 in TIM-4$^+$ BMDMs loaded with dying EL4 cells.

(G) LC3-II levels in TIM-4$^+$ or *Timd4*$^{-/-}$ BMDMs from WT or *Prkaa1*$^{-/-}$ mice loaded with CDDP-treated EL4 cells (EL4 [+]) or not (EL4 [–]).

(H) Localizations of TIM-4 and AMPKα1 in TAMs, TIM-4$^+$ BMDMs, and P-MAC cultured with or without CDDP-treated EL4 cells. See also Figure S4.

TIM-4-Mediated Activation of Autophagy Attenuates Antitumor Effect of Chemotherapy

To examine the involvement of TIM-4-autophagy pathway, we characterized the antitumor effects of CDDP and anti-TIM-4 mAb on MC38 colon cancer growth in the LysM-*Atg5*$^{-/-}$ or control *Atg5*$^{flox/flox}$ mice. The combined treatment with CDDP and anti-TIM-4 mAb resulted in a significant delay of tumor growth in the *Atg5*$^{flox/flox}$ mice (Figure 5A). In contrast, CDDP monotherapy was sufficient to suppress tumor growth in the LysM-*Atg5*$^{-/-}$ mice at a similar extent to combined treatment with CDDP and anti-TIM-4 mAb (Figure 5A). To analyze the mechanisms underlying these effects, we adoptively transferred OT-I cells into LysM-Atg5$^{-/-}$ or control Atg5$^{flox/flox}$ mice. Two days later, B16-OVA cells were inoculated subcutaneously and the mice were then treated with CDDP and/or anti-TIM-4 mAb. Lymphocytes were isolated from the established tumors 7 days after the final treatment. The CDDP alone was sufficient to maximize the frequencies of TCR-Vβ5$^+$ OT-I cells and IFN-γ-producing OT-I cells in the LysM-*Atg5*$^{-/-}$ mice, whereas the combination with anti-TIM-4 mAb was required for maximizing antitumor effects in the control *Atg5*$^{flox/flox}$ mice (Figures 5B and 5C).

Recent studies demonstrated that some chemotherapeutic agents trigger immunogenic cell death (ICD) of tumors, which was associated with tumor immunogenicity and antitumor responses (Green et al., 2009). Because a cytotoxic drug that has been known as a non-ICD inducer (CDDP) was utilized in this study, we next examined how TIM-4-autophagy pathway regulates antitumor responses of ICD inducers (oxaliplatin [OXP], doxorubicin, and γ-irradiation) as compared to non-ICD inducers (CDDP, CPT-11, and mitomycin-C) (Green et al., 2009). Cross-priming of OT-I cells by EG7-loaded BMDMs was significantly augmented irrespective of the frequencies with either ICD or non-ICD inducers (Figure S5B). Although OXP monotherapy substantially suppressed tumor growth in the LysM-*Atg5*$^{-/-}$ mice, the treatment with anti-TIM-4 mAb further augmented antitumor effect of OXP in the *Atg5*$^{flox/flox}$ mice (Figure S5C). Thus, TIM-4-autophagy pathways regulate antitumor responses of chemotherapeutic agents that trigger either ICD or not.

To further define the role of TIM-4 on myeloid cells in regulating antitumor effect of chemotherapy-induced adaptive immunity, we generated mixed BM chimeric mice comprising BMCs from WT or *Timd4*$^{-/-}$ and LysM-Atg5$^{-/-}$ mice. In this setting, BMCs from *Atg5*$^{flox/flox}$, LysM-*Atg5*$^{-/-}$, and *Timd4*$^{-/-}$ or WT mixed with LysM-*Atg5*$^{-/-}$ mice at a 1:1 ratio were used to reconstitute lethally irradiated WT recipient mice. Two

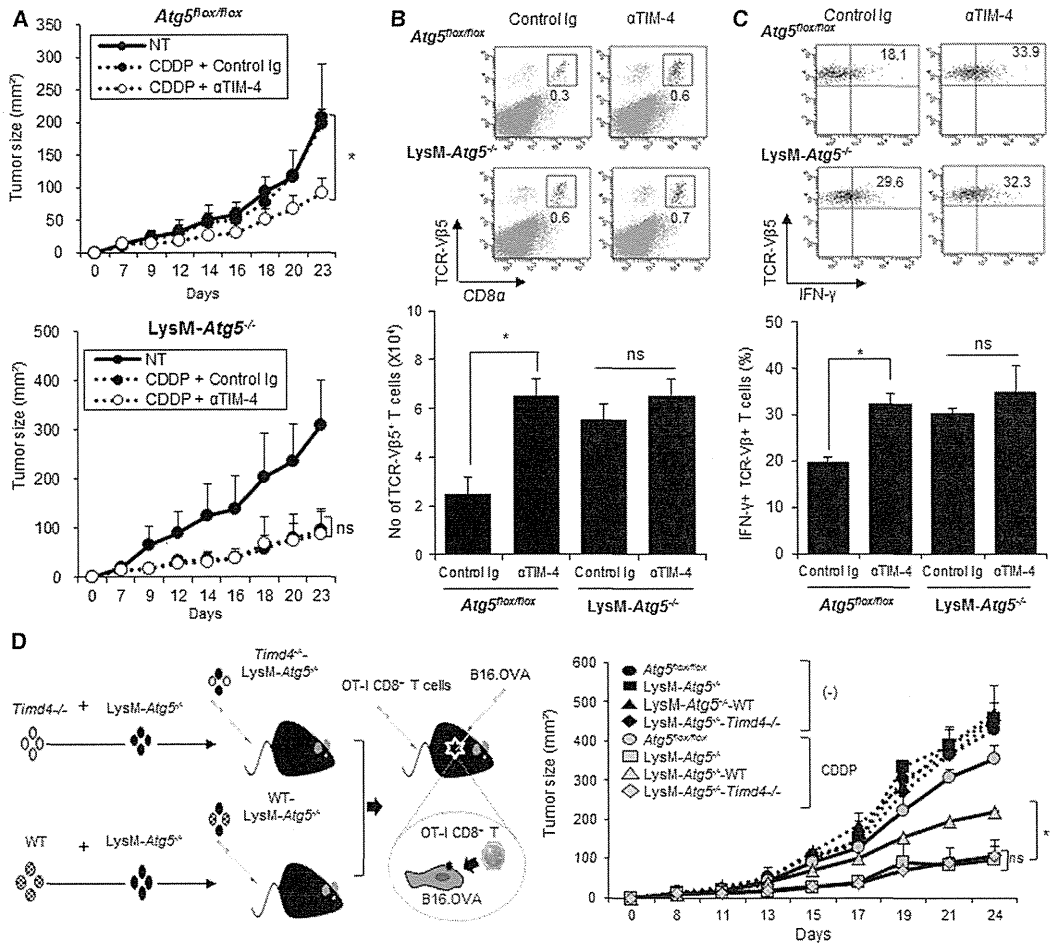

Figure 5. TIM-4-Autophagy Pathway Attenuates Antitumor Effects of Chemotherapy

(A) MC38 tumor growth in LysM-*Atg5*$^{-/-}$ or *Atg5*$^{flox/flox}$ mice treated with CDDP and control Ig or αTIM-4 (n = 4 per group).

(B and C) Total number of CD8$^+$ TCR-Vβ5$^+$ OT-I cells (B) or IFN-γ$^+$ populations of OT-I cells (C) in tumor-infiltrating lymphocytes.

(D) B16-OVA tumor growth in mice reconstituted with BM cells of Atg5$^{flox/flox}$, LysM-Cre-Atg5$^{-/-}$, *Timd4*$^{-/-}$, and LysM-*Atg5*$^{-/-}$ (*Timd4*$^{-/-}$-LysM-Atg5$^{-/-}$) or WT and LysM-*Atg5*$^{-/-}$ (WT/LysM-*Atg5*$^{-/-}$ chimera) mice 3 days after intravenous transfer of OVA-specific TCR-Vβ5$^+$ cells and treated with CDDP. Schematic representation of experimental approach is shown (left panel). Data are shown as mean ± SD (n = 3–5). See also Figure S5.

months after the BMT, OT-I cells were adoptively transferred with B16-OVA tumor cells into the each BM chimeras and treated with CDDP or OXP to induce antigen-specific immune responses in vivo. The antitumor effects of CDDP were compared in *Atg5*$^{flox/flox}$, LysM-*Atg5*$^{-/-}$, *Timd4*$^{-/-}$ -LysM-*Atg5*$^{-/-}$, or WT-LysM-*Atg5*$^{-/-}$ chimeras. CDDP or OXP had a superior antitumor effect on B16-OVA tumors in LysM-*Atg5*$^{-/-}$ BM chimeras compared to *Atg5*$^{flox/flox}$ counterparts. Importantly, the antitumor effect of CDDP or OXP was severely impaired in WT-LysM-*Atg5*$^{-/-}$ BM chimeras compared to LysM-*Atg5*$^{-/-}$ BM mice, whereas both chemotherapies had a potent antitumor effect in *Timd4*$^{-/-}$-LysM-*Atg5*$^{-/-}$BM chimeras at comparable levels to LysM-*Atg5*$^{-/-}$ BM chimeras (Figure 5D; Figure S5C).

Together, these findings further substantiate the role of TIM-4-autophagy pathway in negatively regulating chemotherapy-induced antitumor immunity.

The TIM-4-AMPKα1-Autophagy Pathway Attenuates Antitumor Effects of Chemotherapy

We next evaluated whether the TIM-4-AMPKα1 interaction is responsible for the attenuation of chemotherapeutic effects by TIM-4-mediated activation of autophagy. To do so, TIM-4$^+$ BMDMs were loaded with apoptotic EG7 cells in the presence of AMPK inhibitor (compound C) or activator (5-Aminoimidazole-4-carboxyamide ribonucleoside: AICAR) or transfected with siRNA specifically targeting for AMPKα1 and AMPKα2. The cells were then used to stimulate OT-I cells. TCR-Vβ5$^+$ OT-I cells produced more IFN-γ when stimulated by TIM-4$^+$ BMDMs treated with the AMPK inhibitor or transfected with AMPKα-specific siRNA. Moreover, TIM-4 blockade had little impact on the stimulation by TIM-4$^+$ BMDMs when AMPK was inhibited (Figure 6A). In contrast, the TIM-4 blockade could not overcome the immunosuppressive properties of TIM-4$^+$ BMDMs or decreased the immunogenic potential of TIM-4-deficient

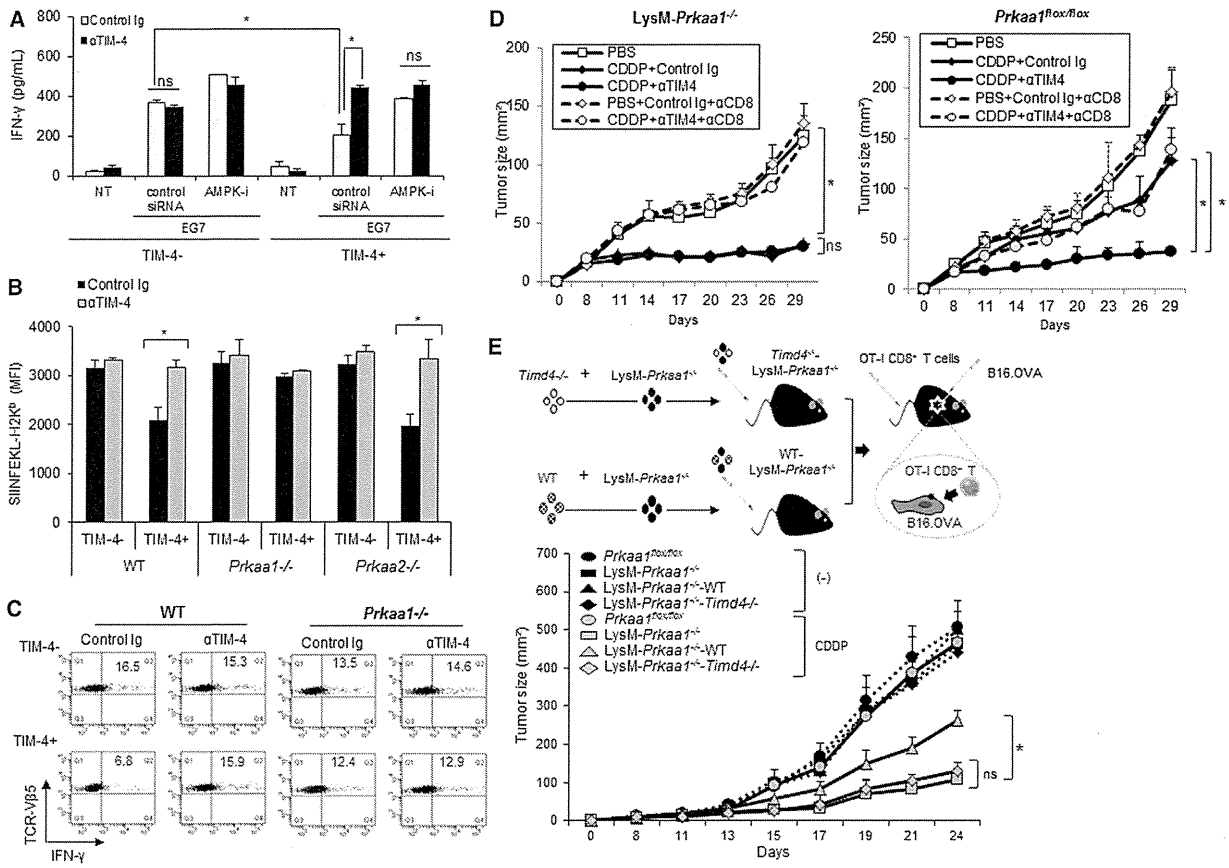

Figure 6. The TIM-4-AMPKα1 Pathway Attenuates Antitumor Effect of Chemotherapy and Tumor-Specific Immune Responses

(A) IFN-γ production of OT-1 cells stimulated by the AMPKα siRNA-transfected TIM-4$^+$ or TIM-4$^-$ BMDMs loaded with CDDP-treated EG7 cells.

(B) OVA presentation of TIM-4$^+$ or TIM-4$^-$ BMDMs from WT, *Prkaa1*$^{-/-}$, or *Prkaa2*$^{-/-}$ mice loaded with CDDP-treated EG7 cells and control Ig or αTIM-4.

(C) IFN-γ production in TCR-Vβ5$^+$ OT-I cells cocultured with TIM-4$^+$ or TIM-4$^-$ WT or *Prkaa1*$^{-/-}$ BMDMs loaded with CDDP-treated EG7 cells.

(D) Subcutaneous MC38 tumor growth in the BM chimeras reconstituted with LysM- *Prkaa1*$^{-/-}$ or *Prkaa1*$^{flox/flox}$ BM cells and treated with CDDP and αTIM-4. For some instances, anti-CD8 depleting Ab was administered 2 days prior to tumor inoculation. (n = 4 per group).

(E) B16-OVA tumor growth in the mice reconstituted with BM cells from *Prkaa1*$^{flox/flox}$, LysM-*Prkaa1*$^{-/-}$, *Timd4*$^{-/-}$, and LysM-*Prkaa1*$^{-/-}$ mice (TIM-4$^{-/-}$-LysM-*Prkaa1*$^{-/-}$ chimera) or WT and LysM-*Prkaa1*$^{-/-}$ mice (WT-LysM-*Prkaa1*$^{-/-}$ chimera) 3 days after intravenous transfer of OVA-specific TCR-Vβ5$^+$ cells from OT-I mice, and treated with CDDP. The schematic representation of experimental approach is shown (top). Data are shown as mean ± SD (n = 3–5). See also Figure S6.

BMDMs when pretreated with the AMPK activator AICAR (Figure S6A). Furthermore, BMDMs and BMDCs generated from *Prkaa1*$^{-/-}$ mice exhibited OVA cross-presentation at greater levels than WT counterparts irrespectively of the treatment with anti-TIM-4 mAb or TIM-4-specific siRNA. In contrast, OVA-presentation was similarly compromised in *Prkaa2*$^{-/-}$ and WT BMDMs in TIM-4-dependent manner, suggesting that α1 subunit of AMPK is critical to interact with TIM-4 and suppress cross-priming by apoptotic cell-derived antigens (Figure 6B; Figure S6B).

Liver kinase B1 (LKB1)-mediated phosphorylation of AMPKα serves as a critical pathway to activate tuberous sclerosis complex (TSC) and suppresses mTOR1 (Alessi et al., 2006). However, TIM-4 activated autophagy and inhibited OVA antigen-presentation in LKB1$^{-/-}$ macrophages at similar levels to WT BMDMs, suggesting that AMPKα1 regulates TIM-4-mediated immune tolerance by LKB1-independent manner (Figure S6C).

To define the role of TIM-4-AMPKα1 pathway in myeloid cells in the regulation of chemotherapy-induced antitumor immune responses in vivo, we generated BM chimeric mice in which BMCs from control *Prkaa1*$^{flox/flox}$ or LysM-*Prkaa1*$^{-/-}$ mice were reconstituted in lethally irradiated WT mice. Two months after the BMT procedure and confirmation of the chimerism by LysM-Cre gene detection, MC38 colon carcinoma cells were inoculated subcutaneously and then treated with CDDP or OXP in the presence of αTIM-4 mAb or control Ig. In this system, we showed the importance of TIM-4-AMPKα1 pathway in myeloid cells in suppressing antitumor effect of OXP, because chemotherapy alone had a remarkable antitumor effect in LysM-*Prkaa1*$^{-/-}$ BM chimeras compared to those in *Prkaa1*$^{flox/flox}$ controls, whereas anti-TIM-4 mAb augmented antitumor effect of CDDP or OXP in the *Prkaa1*$^{flox/flox}$ chimeric mice. More importantly, depletion of CD8$^+$ cells mostly diminished antitumor effects of chemotherapy in LysM-*Prkaa1*$^{-/-}$ BM chimeras, as well as CDDP or OXP

combined with anti-TIM-4 mAb in *Prkaa1*$^{flox/flox}$ chimeras, validating the importance of adaptive immunity in this system (Figure 6D; Figure S6D). The depletion of CD8$^+$ cells had little effect on tumor growth without the treatment with CDDP in our experiments (Figure 6D).

To further clarify the role of TIM-4 on myeloid cells in the regulation of chemotherapy-induced antitumor immunity in vivo, we generated mixed BM chimeric mice comprising of bone marrow (BM) cells from WT or *Timd4*$^{-/-}$- and LysM-*Prkaa1*$^{-/-}$ mice. BMCs from *Prkaa1*$^{flox/flox}$, LysM-*Prkaa1*$^{-/-}$, and*Timd4*$^{-/-}$ or WT mixed with LysM-*Prkaa1*$^{-/-}$ mice at a 1:1 ratio were used to reconstitute irradiated WT recipient mice. Two months after the BMC reconstitution, OT-I cells were adoptively transferred with B16-OVA tumor cells into the each BM chimera and treated with CDDP to evaluate antigen-specific immune regulation. CDDP had a superior antitumor effect in LysM-*Prkaa1*$^{-/-}$ BM chimeras compared to *Prkaa1*$^{flox/flox}$ counterparts. Importantly, the antitumor effect of CDDP was severely impaired in WT-LysM-*Prkaa1*$^{-/-}$ BM chimeras compared to LysM-*Prkaa1*$^{-/-}$ BM mice, whereas CDDP had a potent antitumor effect in *Timd4*$^{-/-}$-LysM-*Prkaa1*$^{-/-}$ BM chimeras at comparable levels to LysM-*Prkaa1*$^{-/-}$ BM chimeras (Figure 6E). Together, these findings indicate that TIM-4-AMPKα1 interaction in myeloid cells attenuates therapeutic effect of chemotherapy by repressing tumor-specific CD8$^+$ T cell immune responses.

DISCUSSION

We have demonstrated in this study that the interaction between TIM-4 and AMPKα1 links phagocytosis of apoptotic cells with immune tolerance. TIM-4 was highly expressed on tumor-associated myeloid cells, which were predominantly recruited from bone marrow, and DAMPs released from dying tumor cells upon cytotoxic chemotherapy-augmented TIM-4 expression on the myeloid cells. TIM-4 on the surface of myeloid cells was translocated to LAMP1$^+$ phagosomes after phagocytosis of apoptotic cells and then interacted with AMPKα1. The TIM-4-mediated activation of AMPKα1 led to formation of autophagic vesicles by activating ULK1 and repressing mTOR signals, which resulted in excess degradation of tumor-cell-derived antigens in autophagolysosomes and thereby suppressed cross-priming of tumor-specific CTL. In this regard, our findings that TIM-4-mediated phagocytosis activates the canonical autophagic pathway might differ from the recently reported LC3-associated phagocytosis, because TIM-4 induced canonical autophagy characterized by ULK1-dependent autophagic vesicle formation was observed following phagocytosis of apoptotic cells. Thus, our findings have revealed the molecular link whereby phagocytosis regulates immune responses to tumor or self-antigens derived from apoptotic cells, and targeting TIM-4-AMPKα1 pathway provide a novel strategy for augmenting immunogenic cell death upon chemotherapy while attenuating autoimmunity.

We observed that TIM-4 expression was significantly upregulated on macrophages and DCs in the tumor microenvironments and demonstrated that DAMPs such as HMGB1, HSP90, MSU, S100A8, and ATP released from damaged tumor cells were responsible for upregulating TIM-4 expression on macrophages. Because TIM-4 mediates phagocytic clearance of apoptotic cell debris, the TIM-4 induction by DAMPs released from dying cells might be relevant in physiological conditions. Given the immunosuppressive function of TIM-4 on APCs as described in this study, it will be of great interest to examine the expression of TIM-4 in various diseases such as chronic infections and autoimmune diseases, in which DAMPs are implicated in the pathogenesis. DAMP-mediated induction of TIM-4 on macrophages may not only facilitate the clearance of damaged cells but also dampen autoimmune responses by suppressing cross-priming with self-antigens. This notion is consistent with the development of autoimmunity in TIM-4-deficient mice (Rodriguez-Manzanet et al., 2010).

Autophagy either promotes or suppresses tumor growth depending on oncogenic and metabolic alterations in different tumor cells and their microenvironments (Kimmelman, 2011; White, 2012). In addition, cytotoxic chemotherapy triggers cell death pathways by activating autophagic machineries, whereas autophagy protects tumor cells from cell death triggered by chemotherapy in certain circumstances (White, 2012). Thus, molecular mechanisms by which autophagic pathways differently regulate antitumor effects of cytotoxic chemotherapy remain largely elusive. Our findings of TIM-4-mediated degradation of tumor antigens by autophagy provide evidence that autophagic pathways might be involved in immunosuppressive property of chemotherapy under the conditions that TIM-4-expressing myeloid cells are abundantly infiltrated into the tumor microenvironments.

Accumulating evidences have revealed that the receptor-mediated uptake of apoptotic cells modulates multiple signaling cascades, such as those regulating cytoskeletal organization, energy, and immune homeostasis (Nagata et al., 2010). Although LC3-associated phagocytosis is frequently observed upon recognition of apoptotic cells, it remains largely unknown whether the recognition of apoptotic cells by some phagocytic receptors directly induce autophagic vesicle formation and activation of conventional autophagy. Contrary to the role of autophagy as a critical defense system to prevent microbes from evading phagosomal degradation (Levine et al., 2011), we demonstrate that autophagic degradation of ingested tumor cells might cause a detrimental effect on antitumor immunity by abolishing immunogenic antigens available for stimulating tumor-specific CTLs. Thus, temporal and spatial cooperation of phagocytosis and autophagy-mediated overdegradation of tumor antigens might constitute a critical determinant for tumor evasion from immunosurveillance after cytotoxic chemotherapy. Consistent with this notion, we have demonstrated here that blockade of the TIM-4-AMPKα1-autophagy pathway significantly augmented the antitumor effects of chemotherapy in CTL-dependent manner.

Although AMPK serves as an energy checkpoint in cellular metabolism and homeostasis, recent studies unveiled the role of AMPK in regulation immune functions of various lymphocyte subsets, including differentiation of memory CD8$^+$ T cells and tolerance induction by APCs (O'Neill and Hardie, 2013). In this study, we provided evidence that AMPKα1 mediates immune tolerance with TIM-4 in LAMPK1$^+$ phagosome upon ingestion of apoptotic cells, which leads to ULK1 phosphorylation and autophagy activation.

Although AMPK activator such as antidiabetic drug metformin plays an essential role in reprogramming metabolic profiles in

cancer cells, it is emerging as a new therapeutic option for malignant diseases (Gallagher and LeRoith, 2011; Faubert et al., 2013). In marked contrast, our findings revealed that TAMs impede anticancer effects of cytotoxic chemotherapy through TIM-4-AMPKα1 pathway in myeloid cells. Thus, it is critical to clarify the optimal conditions in which particular types of anticancer drugs should be combined with AMPK activator to avoid TIM-4-mediated immune tolerance. In addition, AMPK activator might have an additional role to control autoimmunity such as diabetes by suppressing immunogenic antigen presentation when TIM-4 on myeloid cells recognizes apoptotic cells. Together, these multifaceted properties of AMPK highlight the need for a more deep understanding of how AMPK regulates tumor environments, which will be essential to develop drugs targeting AMPK-related signals to optimize their therapeutic potential to inflammatory disorders such as cancer, autoimmunity, and chronic infection.

Different subsets of macrophages are characterized by phenotypic diversity, anatomical location, and their functions (Geissmann et al., 2010). We show here the distinct properties of myeloid cells from peritoneal cavity, bone marrow, and tumor tissues in the context of TIM-4-mediated regulation of phagocytosis and antigen presentation. These differences relied mainly upon the differential potential of AMPKα1 activities in each macrophage subset by specific environmental stress, because TIM-4 was preferentially interacted with AMPKα1 in TAMs than peritoneal macrophages after engulfment of dying tumor cells. These findings revealed that a different threshold of particular molecular signals in the macrophage subsets might serve as a determinant factor to control quality and intensity of local immune responses when encountered with potential immunogenic materials.

In summary, our present study has revealed a mechanism for the tolerogenic property of apoptotic cells through TIM-4-mediated activation of AMPKα1 in phagocytic APCs, which compromised the immunogenic antitumor effects of chemotherapy. Thus, targeting of the TIM-4-AMPKα1 pathway would be also effective to augment antitumor immune responses after general cytotoxic cancer therapies such as radiation, immunotoxins, and adaptive transfer of CTLs. In addition, the TIM-4-AMPKα1 pathway might also be involved in the maintenance of peripheral immune tolerance and the pathogenesis of autoimmune diseases. Further studies are needed to address these possibilities.

EXPERIMENTAL PROCEDURES

TIM-4 Expression on Myeloid Cells

TIM-4 expression on macrophages prepared from tumors or normal tissues of tumor-bearing mice was analyzed by flow cytometry with a mAb against mouse TIM-4 (RMT4-53). In tumor tissues obtained from cancer patients, the TIM-4 levels on macrophages or DCs were also examined by flow cytometry with an anti-human TIM-4 mAb.

In Vitro Cross-Priming Assay

TIM-4$^+$ WT or *Timd4*$^{-/-}$ cells were cocultured with chemotherapy-treated tumor cells for 4 hr, and then the cells were subjected to antigen presentation analysis by quantifying OVA peptide (SIINFEKL)-H2Kb complex on the cell surface with 25-D1.16 mAb. For some instances, the cells were pretreated with various inhibitors or activators (see Supplemental Experimental Procedures) for 4 hr before loading with the tumor cells. Intracellular IFN-γ expression in TCR-Vβ5$^+$ OT-I cells was determined by flow cytometry.

In Vivo Tumor Studies

WT, LysM-*Atg5*$^{-/-}$ *Atg5*$^{flox/flox}$, LysM-*Prkaa1*$^{-/-}$, or *Prkaa1*$^{flox/flox}$ mice were injected subcutaneously with 1×10^5 live tumor cells. Chemotherapeutics (see Supplemental Experimental Procedures) were administered i.p. to the mice on days 8, 10, and 12 in the presence of anti-TIM-4 mAb or isotype-matched control Ig. Tumor growth was measured on the indicated days.

TIM-4-AMPKα1 Interaction

The interaction between TIM-4 and AMPKα1 in macrophages loaded with chemotherapy-treated tumor cells was evaluated by immunoprecipitation and immunofluorescence microscopy (see Supplemental Experimental Procedures).

Statistics

The differences between two groups were determined by the Student's t test or the two-sample t test with Welch's correction. The differences among three or more groups were determined by a one-way ANOVA. p values less than 0.05 are considered statistically significant. * $p < 0.05$, ns: not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.09.014.

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REVIEW

The impact of the TIM gene family on tumor immunity and immunosuppression

Muhammad Baghdadi[1] and Masahisa Jinushi[2]

Tumor immunoevasion is an advanced phase of cancer immunosurveillance in which tumor cells acquire the ability to circumvent host immune systems and exploit protumorigenic inflammation. T-cell immunoglobulin mucin (TIM) gene family members have emerged as critical checkpoint proteins that regulate multiple immune response phases and maintain immune homeostasis. Accumulating evidence demonstrates that tumor cells exploit TIM gene family members to evade immunosurveillance, whereas TIM gene family members facilitate the prevention of inflammation-related tumor progression. Thus, a comprehensive analysis to clarify the relative contributions of TIM gene family members in tumor progression may elucidate immunosurveillance systems in cancer patients.
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Keywords: antitumor immunity; immunoevasion; immunosurveillance; TIM; tumorigenic inflammation

INTRODUCTION

The interaction between tumor cells and host immune cells plays an important role in multiple stages of tumorigenesis, and recent clinical evidence suggests a potential contribution of host immune responses in modulating the clinical outcome of cancer patients.[1,2] Moreover, manipulation of the endogenous immune system has emerged as an effective anticancer therapy in patients with advanced cancer.[3,4]

Interestingly, accumulating evidence has revealed that the tumor microenvironment has a significant impact on the functional properties of certain immunoregulatory components that regulate whether host responses promote or antagonize tumor growth. Tumor cells and tumor-infiltrating lymphocytes adopt strategies to evade antitumor processes and may enhance the metastatic potential through the activation of chronic inflammatory signals.[5,6] Together, these observations underscore the complexity of host immune system regulatory pathways in the regulation of tumorigenesis.

In this article, we describe the potential impact of the T-cell immunoglobulin mucin (TIM) gene family in tumor immunosurveillance and immunoevasion and the impact of different tumor microenvironments on the therapeutic responses of TIM-targeted therapies.

MECHANISMS OF TUMOR IMMUNOSURVEILLANCE AND IMMUNOEVASION

Transformation is established by overcoming multiple intrinsic and extrinsic tumor suppression mechanisms. Transformed cells are detected intrinsically using checkpoint mechanisms that survey genetic and epigenetic abnormalities such as oncogene-induced senescence, DNA damage responses or apoptotic/necrotic cell death programs.[7] Extrinsic tumor surveillance systems detect transformed cells by utilizing non-transformed cells within tumor microenvironments. In particular, the innate and adaptive immune systems play a critical role in detecting and eliminating transformed cells by activating multiple sets of myeloid cells and lymphocytes.[2] Interestingly, tumor-infiltrating immune cells also contribute to tumor progression by triggering tumor angiogenesis and immune suppression.[8–10] These findings suggest that the host immune system contributes to tumor initiation and progression in a contradictory manner.

Although the mechanisms that regulate tumor immune responses require further clarification, the recent concept of 'cancer immunoediting' might explain the differential temporal and spatial dynamism of tumor immunosurveillance and immunoevasion, as evidenced by the antitumorigenic and protumorigenic host immune responses during different phases of tumorigenesis. Classically, cancer immunoediting has been

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divided into three phases: elimination, equilibrium and escape. In the elimination phase, innate and adaptive lymphocytes detect the presence of transformed cells and remove them. However, tumor immunosurveillance selectively eliminates highly immunogenic transformed cells, while poorly immunogenic cells survive and enter into the equilibrium phase. The interaction between surviving tumor variants and immune cells creates a homeostasis in which low-level malignant and/or quiescent tumor cells coexist with lymphocytes. After a long-term equilibrium between tumor cells and lymphocytes, additional genetic and epigenetic alterations allow tumor cells to evade tumor immunosurveillance. During this immunoevasion phase, tumor cells not only circumvent antitumor immunity, but also promote tumorigenic activities through multiple tumor-intrinsic and extrinsic machineries.[8,10,11] These immunoevasion mechanisms elicited by the tumor microenvironment include the secretion of immunosuppressive cytokines, the emergence of tumor antigen-loss variants and the subversion of antigen-specific CTL responses by immunosuppressive antigen-presenting cells (APCs).[12–16]

Moreover, the tumor microenvironment adopts multiple strategies to subvert tumor immunosurveillance by recruiting immunosuppressive myeloid cells, such as IDO$^+$ dendritic cells (DCs), B7-1$^+$ macrophages, angiogenic DCs and Foxp3$^+$ regulatory T cells (Tregs), which severely compromise endogenous immunogenicity and impair therapeutic responses to immunotherapy.[17–19] More importantly, tumor cells exploit host immunity to create intimal networks among tumor cells, stromal cells and endothelial cells and to promote pro-tumorigenic inflammation-associated carcinogenic responses such as angiogenesis and epithelial–mesenchymal transitions.[20,21] Tumor immunoevasion has multiple impacts on tumorigenesis by triggering tolerogenic responses to tumor cells and by exploiting pro-tumor inflammation.

THE ROLE OF TIM MOLECULES IN THE REGULATION OF IMMUNE HOMEOSTASIS

TIM proteins are type-I cell-surface glycoproteins composed of a signal peptide, an extracellular IgV domain, a mucin-like domain, a transmembrane domain, and an intracellular cytoplasmic tail. All members share a conserved sequence homology in the IgV domains, while other domains demonstrate poor homology (Figure 1). Under healthy conditions, the TIM family is largely confined to restricted sets of lymphoid and myeloid lineage cells and kidney epithelial cells. However, the induction of TIM gene family members is frequently observed on cell types such as stromal cells, endothelial cells and transformed cells in chronic viral infections and cancer, implying that TIM proteins play a critical role in the regulation of these pathological conditions.[22,23]

Moreover, the TIM gene family has multiple roles in the regulation of immune activation and tolerance, which may have a large impact on the clinical consequences of sterile inflammation, antimicrobial defense and antitumor immune responses.[24,25] For example, continuous blockade of TIM-3 causes autoimmune nephritis in immune competent animals, and TIM-3 triggers antimicrobial immunity by interacting with galectin-9[26] and impedes innate antitumor responses.[27,28] Moreover, TIM-1 promotes pro-inflammatory responses that may be associated with aggressive graft-*versus*-host disease.[29] Thus, these findings raise the possibility that TIM family members serve as critical checkpoints to regulate immune homeostasis and inflammation. More importantly, accumulating evidence has unveiled the critical roles of TIM family members in the regulation of antitumor immunosurveillance. Thus, we will provide an overview of TIM-mediated regulation of tumor immunity and perspectives on the potential of TIM family members for tumor immunosurveillance and immunoevasion.

THE ROLE OF TIM MOLECULES IN THE IMMUNE REGULATION OF TUMORS

TIM-1

TIM-1 is mainly expressed on T cells and kidney epithelial cells.[24,25] Several lines of evidence suggest a dual role for TIM-1 in the regulation of T cell-mediated immunity. TIM-1 may act as a costimulatory molecule for T-cell activation [30,31] or transduce a negative signal that leads to the inhibition of T-cell effector function.[32] Several studies have reported multiple roles for TIM-1 in creating immunostimulatory or immunosuppressive environments. For example, treatment with anti-TIM-1 antibodies in the effector phase impedes the development of inflammation, while treatment during the priming phase results in a break in immune tolerance.[31–33] In addition, TIM-1 on the surface of macrophages acts as a phosphatidylserine receptor that phagocytoses apoptotic cells.[34] Thus, it is highly likely that TIM-1 play a role in the efficient clearance of apoptotic cells and the maintenance of tumor microenvironment homeostasis.

The molecular mechanism of TIM-1-mediated immune regulation has been recently demonstrated. In this study, TIM-1 recycles from the cell surface to endosomes using clathrin-dependent endocytosis pathways, and it suppresses the expression of the orphan nuclear receptor NUR77 through lysosomal degradation pathways, thus protecting kidney epithelial cells from NUR77-mediated apoptotic death signals.[35] NUR77 also serves as a lineage-specific factor that promotes monocyte differentiation from BM precursors.[36] Additionally, NUR77 inhibits pro-inflammatory activities by myeloid cells in murine atherosclerosis models.[37] Thus, NUR77 may contribute to the creation of tolerogenic microenvironments by generating immunosuppressive myeloid cell lineages. It is tempting to speculate that TIM-1 generates tolerogenic myeloid cells that promote pro-inflammatory responses by interfering with NUR77-dependent immunosuppressive programs (Figure 2).

TIM-1 has multiple functions in the regulation of tolerance and immunity depending on the cell type and microenvironment; it remains unclear whether the functional multiplicity of TIM-1 could affect the direction and quality of antitumor immunity and tumor-associated inflammation (Figure 3).

TIM-2

TIM-2 is preferentially expressed in differentiated Th2 cells.[24,25] TIM-2 blockade results in T cell hyperproliferation

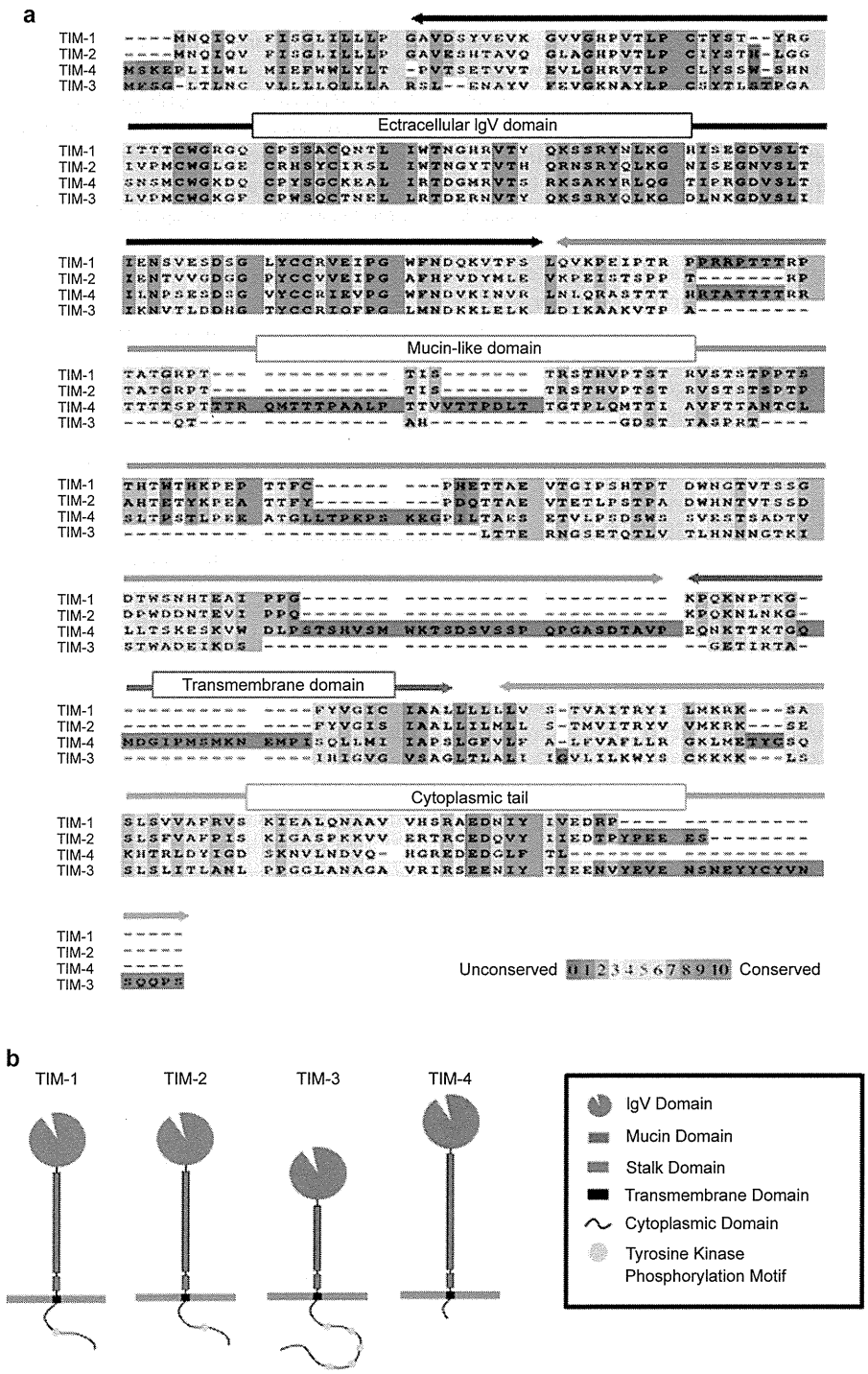

Figure 1 The structure and main domains of TIM gene family members. The sequences (**a**) and schematic structures (**b**) are shown for each TIM gene family member. TIM molecules are type-I cell-surface glycoproteins that comprise an extracellular IgV domain, a mucin-like domain, a transmembrane domain and an intracellular cytoplasmic tail. All TIM molecules have a conserved sequence homology in the IgV domains, while other domains show little similarity. The sequence data were generated using the PRALINE multiple sequence alignment function. TIM, T-cell immunoglobulin mucin.